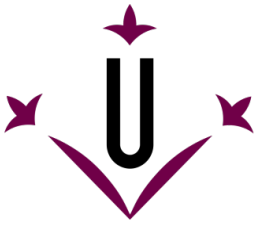


Response profile of pheromone receptor neurons in male *Grapholita molesta* (Lepidoptera : Tortricidae)

Byrappa Ammagarahalli, César Gemeno

Department of Crop and Forest Sciences, University of Lleida, 25198, Lleida, Spain

Corresponding author Tel.: +34 (973)702531; fax: +34 (973)238264.

E-mail addresses: nnnbyrareddy20@gmail.com (B. Ammagarahalli), cesar.gemeno@pvcf.udl.cat (C. Gemeno)

abstract

The response profile of olfactory receptor neurons (ORNs) of male *Grapholita molesta* (Busck) to the three female sex pheromone components [(*Z*)-8-dodecenyl acetate (*Z*8-12:Ac), (*E*)-8-dodecenyl acetate (*E*8-12:Ac) and (Z)-8-dodecenyl alcohol (*Z*8-12:OH)] was tested with single sensillum electrophysiology. Sensilla trichoidea housed normally one, but sometimes two or three ORNs with distinct action potential amplitudes. One third of the ORNs contacted were unresponsive to any of the pheromone components tested. The remaining ORNs responded either to the major pheromone component, *Z*8-12:Ac (63.7%, so called "Z-cells"), or to its isomer *E*8-12:Ac (7.4%, so called "E-cells"), but none responded to *Z*8-12:OH. Z- and E-cells were housed in separate sensilla trichodea. The proportion of Z- and E-cells on the antennae (100:16, respectively) is similar to the proportion of these compounds in the blend (100:6, respectively). The response of Z-cells was very specific, whereas E-cells also responded to the Z isomer, albeit with lower sensitivity.

Keywords: *Grapholita molesta*, single sensillum recording, sex pheromone, olfactory receptor neuron, sensillum

1. Introduction

Grapholita molesta (Busck) larvae bore on new growth shoots of peach trees (*Prunus* spp.) reducing fruit yield (Rothschild and Vickers, 1994). The sex pheromone has been described as a 100:6:10 blend of (*Z*)-8 dodecenyl acetate (*Z*8-12:Ac), (*E*)-8 dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8 dodecen-1-ol (*Z*8-12:OH), respectively (Roelofs et al., 1969; Beroza et al., 1974; Cardé et al., 1975a; Cardé et al., 1979; Baker and Cardé, 1979; Baker et al., 1981; Linn and Roelofs, 1983), and is used for monitoring and mating disruption over 50,000 hectares of peach and apple around the world (Witzgall et al., 2010).

The behavioural response of *G. molesta* to pheromone and plant odours has been studied in detail (e.g., Linn and Roelofs, 1981; Willis and Baker 1988; Linn et al., 1988; Linn et al., 1991; Willis and Baker, 1994; Piñero and Dorn, 2007a; 2009; Ilichev et al., 2009; Varela et al., 2011b; Lu et al., 2012; 2013; Najar-Rodriguez et al., 2012; 2013; Trimble, 2012). Electroantennography has been used to explore questions mainly related to mating disruption (e.g., Stelinski et al., 2006; Molinari et al., 2010; Trimble and Marshall, 2010; Khuns et al., 2012; D'Errico et al., 2013; Faraone et al., 2013), and at the CNS level, the tridimensional structure of the antennal lobe (AL), and the physiological response of AL neurons to pheromone and plant odours have been studied (Najar-Rodriguez et al., 2010; Varela et al., 2011a). In addition Nagy and George (1981) and George and Nagy (1984) described the neuroanatomy of sensilla and olfactory receptor neurons in males, and Baker et al. (1988) analysed the effect of temperature on the ability of olfactory receptor neurons (ORNs) to detect pheromone pulses. However, a detailed characterization of the physiological response of pheromone receptor neuron types in *G. molesta* is lacking.

Pheromone ORNs make a large percentage of the receptors on the male moth antenna and are extremely sensitive to low doses of sex pheromone (Kaissling, 2004). Electrophysiological studies in moths show specific pheromone component detection by distinct ORNs, which may be housed singly in a sensillum trichodeum or paired with other pheromone ORNs (reviewed by De Bruyne and Baker, 2008; and Baker et al., 2012). In general, there is a correlation between the proportion of ORNs that respond to the major and minor pheromone components and the relative abundance of these compounds in the female-produced sex pheromone blend (Baker et al., 2012).

The aim of our study was to characterize the physiological response of male *G. molesta* ORNs to the three components of the sex pheromone blend. We mapped the position of different sensillum types on the antennae by SEM, and recorded the response of ORNs housed in sensilla trichodea to several doses of the pheromone components. We expected that males of *G. molesta* have ORNs specific for each of the three pheromone components and that these are present in a proportion similar to the proportion of the pheromone components in the pheromone blend. Similarly, we expected that these ORNs would be highly sensitive and specific to their respective ligands.

2. Methods

2.1. Insects

The *G. molesta* colony originated from a laboratory colony established at Piacenza, Italy, from insects collected in peach orchards in that locality, and was maintained at the University of Lleida, Spain, since 2005. Larvae were reared on a semi-synthetic diet modified from Ivaldi-Sender (1974) under a L16:D8 photoperiod at 25 ± 1º C. Pupae were separated by sex and were placed in 4-L polypropylene containers provided with a cotton ball soaked in 10% sugar dissolved in water. Adults were separated daily and used when 2-4 days old. Care was taken not to expose adults to synthetic odour sources before the studies.

2.2. Scanning electron microscopy (SEM)

Male antennae were excised from the head with fine forceps. Scales were removed individually by hand under the stereomicroscope using a sharpened tungsten electrode, watching not to damage the sensilla hidden underneath. Antennae were mounted on SEM stubs lined with conductive double-side adhesive black tape, with the orientation of the mounted antenna to show the areas of interest. Preparations were air dried at room temperature for 3-4 days and then coated using a sputter coater (Balzers SCD 050, Leica Microsystems, Spain) with 50-nm gold particles for 3 min from a distance of 50 mm, with a current of 45 mA and Argon as cooling gas. Samples were examined in a scanning electron microscope (DSM 940A, Zeiss, Germany) at 10 Kv and a working distance of 12 mm. Four scale-free and 9 scale-covered antennae from different individuals were examined. Sensilla counts were made on the scaled and scale-free areas of the antennae every 5th flagellomere, starting on the first proximal one. Total sensilla count per antennae was estimated by extrapolating these counts to the

other flagellomeres. The scale-free area, which covers one third of the perimeter of each flagellomere, was fully visible, but the scaled area, which covers the remaining of the surface, was always partially obstructed from vision. Using characteristic landmark structures that indicated the sagital axis on the scaled area we could extrapolate sensilla counts from the visible section of the scaled area to the section hidden from view. Abundance and pattern of distribution of all types of sensilla are reported. Length, basal and tip width of all types of sensilla (N = 20 sensilla from four different antennae) were measured.

2.3. Odourant stimuli

The pheromone compounds of *G. molesta*, (*Z*)-8-dodecenyl acetate (*Z*8-12:Ac), (*E*)-8-dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8-dodecen-1-ol (*Z*8-12:OH) were provided by Pherobank (The Netherlands) with an initial purity ≥ 99%. Gas chromatographic analysis revealed that *Z*8-12:Ac contained 0.38 % *E*8-12:Ac, and that *E*8-12:Ac contained 0.24% *Z*8-12:Ac. Undiluted compound was weighted and diluted in *n*-hexane to make 100 μg/μl stock dilutions. Serial 10-fold dilutions of the stock dilutions in *n*-hexane were prepared from the stock solution as needed.

2.4. Electrophysiological recordings

Males were immobilized with industrial grade CO_2 for 10 s, and were mounted on a handcrafted poly(methyl methacrylate) insect holder. The body was inserted through a hole drilled in the holder and the protruding head was restrained by fixing a piece of adhesive cloth tape between the head and the holder. The antennae were carefully laid on a slant surface lined with double sided sticky tape, and were oriented for easy access with the electrodes. To record from sensilla located on the scaled area, which accounts for 70% of the antennal surface, scales were removed by gently rolling

the antennae on the sticky tape. Remaining scales were removed individually with the help a tungsten electrode. Sub-milimetric smoking paper strips placed over the antennae and glued to the sticky surface prevented antennal torsion. A stereo microscope (objective 2x, oculars 25x, zoom range 0.8-12.5, Leica, Madrid, Spain) was used to help in these operations and to visualize the recordings. These were obtained by means of electrolytically (20% KNO_2) sharpened tungsten microelectrodes (0.125 mm, 99.98% purity, Advent Research Materials Ltd, England). The reference electrode was inserted in the head through the mouth parts. For electroantennogram recordings (EAG) the tip of the recording electrode was inserted in one of the most distal segments of one antenna. For single sensillum recordings (SSR) the recording electrode was situated near the base of a randomly chosen sensillum trichoidea with the help of a manual micromanipulator (NMN-25, Narishige, Japan) and pushed gently inward until action potentials were detected. The signal from the recording electrode was pre-amplified (Universal Single Ended Probe, Syntech, Germany), filtered and digitalized (IDAC-4, Syntech, Germany), and recorded and analyzed in a PC (AutoSpike v.3.9, Syntech, Germany). Flagellomeres 10^{th} to 35^{th} were sampled. The setup was mounted on an anti-vibration table (63-511, TMC vibration control, USA) and was shielded by a grounded metal screen case to reduce low frequency noise.

2.5. Odour stimulation

Dilutions were applied as 1μl aliquots (1μl micropipettes, Drummond Scientific Co., USA) on 1 x 20 mm *n*-hexane-pre-cleaned filter paper strips (# 1, Whatman International Ltd, England). After dry (5 min) the filter papers were introduced in *n*-hexane-pre-cleaned 100 μl glass micropipettes (1.2 mm internal diameter, BlaubrandR Intramark, Germany) which were then placed in glass test tubes sealed with PTFE-

coated screw caps (International Burolab, Barcelona). New stimuli cartridges were prepared each day, and a given cartridge was not used for more than 10 stimulations per day. Air flow was generated by two diaphragm aquarium pumps connected to a 3-way solenoid valve (CS-55, Syntech, Germany). A 0.5 l/min flow of charcoal filtered and humidified air was blown continuously over the insect preparation through a 5-mm internal diameter plastic tube placed 15-20 mm from the preparation (air velocity at exit = 0.4 m/sec). The tip of the odour cartridge bearing the filter paper was positioned 0.4 mm down from the recording point and perpendicular to the direction of the continuous air flow. A 0.2 l/m charcoal-filtered room air flow was puffed through the odour cartridge to the recording area for 200 ms (air velocity at exit = 2.9 m/sec). Time interval between puffs was at least 60 s, but longer if needed to let the spike activity return to pre-stimulation levels. A maximum of 5 cells were recorded per insect, and at least 30 min between two cell recordings were allowed. Test tubes were rinsed with acetone and baked at 250°C overnight before reused.

2.6. Dose-response

Preliminary tests determined the range of concentrations to be used in the dose-response tests. For the EAG we used 10, 100 and 1000 ng of each pheromone compound. For SSR the ORNs were first challenged with a high dose of each pheromone component (100 pg of *Z*8-12:Ac, 1 ng of *E*8-12:Ac, and 1 ng *Z*8-12:OH) to determine their physiological type (cells were typically more sensitive to one pheromone component and less sensitive to the others, see Results), and then dose-response curves were established in ORNs with stable contacts and good signal to noise ratio. The order of stimuli was first the negative control (solvent), followed by low to high doses of the test compounds. For each cell the full dose range of concentrations

was tested for the most sensitive compound, whereas the range of concentrations tested for the other two compounds depended on the cell type. "Z-cells" were very sensitive and specific to *Z*8-12:Ac and were challenged with the full range of concentrations of *Z*8-12:Ac, but only with the two highest dosages of the other two compounds (except for a subset of cells that were tested with the full range of *E*8-12:Ac). "E-cells" were most sensitive to *E*8-12:Ac, and moderately sensitive to *Z*8-12:Ac, so they were tested with the full concentration range of the two acetate isomers, but only with the two highest concentrations of the alcohol. Non-linear regression functions were fitted to the observed data (Byers, 2013). To correct for the 0.38% of *E*8-12:Ac present in *Z*8-12:Ac the regression function of E-cells stimulated with *E*8-12:Ac was used to estimate how much of the response of the E-cells to stimulation with *Z*8-12:Ac was due to the *E*8-12:Ac contaminant, and the estimated response to the contaminant was subtracted from the observed response to the target compound. Non-linear regression parameters were calculated using self-starting functions in R software (Crawley, 2009; R Core Team, 2012)

2.7. Cross-adaptation

A cross-adaptation test was performed to determine if the dual response of E-cells to *E*8-12:Ac and *Z*8-12:Ac was the result of a) one ORN responding to both compounds, or b) two ORNs of equal spike amplitude sharing the same sensillum but each responding to one of the two isomers. Two pheromone cartridges were angled at 45 degrees of each other with the vertex pointing to the SSR preparation. Each cartridge was connected to a different air flow and solenoid valve so that they could puff independently with the same airflow conditions described above. Once contact was established with an ORN it was first stimulated with *Z*8-12:Ac and *E*8-12:Ac to

determine its type (Z- or E-cell, see Results). For cross-adaptation a single 200-ms puff from the first cartridge was followed by a 100-ms inter-stimulus interval and then by a 200-ms puff from the second cartridge. All possible combinations of the E- and Z-isomers (E and E, Z and E, E and Z, Z and Z) were tested in a given cell. The position (left or right pipette) of the stimulus was randomized among replicates. *Z*8-12:Ac and *E*8-12:Ac were puffed at 100 pg and 1ng, respectively, because these concentrations resulted in similar spike frequency responses according to the dose-response curves on E-cells.

2.8. Spike analysis

When more than one spike size was detected they were sorted by amplitude. For each puff the number of spikes during 1-sec pre-stimulation period was subtracted from the number of spikes during 1-sec post-stimulation period. Peri-stimulus time histograms (PSTH) were plotted by grouping spikes in 25 ms bins starting at the onset of stimulation.

The response to *Z*8-12:Ac was more tonic in Z-cells than E-cells. To determine if this difference was significant we calculated the times of half-rise and half-fall peak response, relative to the spontaneous activity (averaged for 1 sec pre-stimulation). PSTHs of Z cells and E-cells to 100 pg and 1 ng loads of *Z*8-12:Ac, respectively, were normalized relative to the peak response, and 2nd order polynomial equations were fitted to the rise and fall phases. Estimated half-rise and half-fall times of cells stimulated with *Z*8-12:Ac were compared between Z- and E-cells with t-tests.

3. Results

3.1. Morphology

The antenna of male *G. molesta* males is filiform and consists of 2 basal segments, scape and pedicel, and a flagellum composed of 45 flagellomeres, with no variation in number of flagellomeres among the 13 antennae from 13 different males analyzed. The flagellum carries most of the sensilla on the antenna. The dorsal and lateral areas of the flagellum bear scales, while a ventral band running the entire length of the flagellum (about 30% of the flagellomeres surface) remains scale-free. The apical flagellomere does not bear any scales.

Scanning electron microscopy of antennae revealed 6 different types of sensilla on the flagellomeres: trichodea, chaetica, coeloconia, auricillica, basiconica and styloconica (Fig. 1). The different types varied in distribution and density along the flagellum and between scaled and scale-free areas (Fig. S1). Sensilla trichodea were thin and long, (Table S2) and were surrounded by a socket-like structure. There were 2291 sensilla trichodea per antennae, which constituted 72% of all the sensilla (Table S1). Their number increased steeply between flagellomeres 1 and 5, remained high between flagellomeres 5 and 35, and decreased steeply from flagellomere 35 towards the distal end of the antenna (Fig. S1). The average number of sensilla trichodea per flagellomere in flagellomeres 5 to 35 was similar in the scaled and scale-free areas (mean ± SEM, 34.9 ± 0.4 and 34.9 ± 0.6, respectively), and therefore sensilla trichodea were denser in the smaller scale-free area than in the twice larger scaled area (Table S1, Fig. 1). Spatial distribution of sensilla trichodea in a flagellomere was random in the scale-free area, and arranged in rows in between the scales in the scaled area (Fig. 1). The second most abundant type of sensilla in the antennae of *G. molesta* males was sensilla auricillica

(Table S1, Figs. 1 and S1). These are much shorter than sensilla trichodea and flattened rather than cylindrical, with a more variable range of sizes and shapes than sensilla trichodea (Table S2, Fig. 1). Sensilla auricillica constituted 11% of the total sensilla in the antenna and were more abundant in the scaled area than in the scale-free area (Table S1, Figs. 1 and S1). They always occurred on the distal area of the flagellomere, being more numerous on the lateral sides of the scaled area, less numerous on the scale-free area, and lacking in the central section of the scaled area (Fig. 1). The third most abundant type of sensilla (7% of the total sensilla) were the coeloconica (Table S1). These are easy to recognize by the central dome surrounded by 12-13 finger-like projections (microtrichia) (Fig. 1). Their distribution overlapped with that of the sensilla auricillica. Sensilla basiconica made only 4 % of the total sensilla. They look similar to sensilla trichodea but are comparatively shorter and wider at the base, and lack the socket-like structure of the trichoid sensilla (Table S1). They are present both in scaled and scale-free areas of the antennae.

The remaining two types of sensilla, chaetica and styloconica, were present in constant number and position in each flagellomere and served as topographic landmarks (Fig. 1). Each flagellomere, except the apical, bears one sensillum styloconica at the distal end of the mid-ventral area (Fig. 1). It consists of a finger-like structure, with a large pore at the terminal end. Sensilla chaetica are similar to sensilla trichodea but they can be distinguished from the former ones because sensilla chaetica have a bulbous socket at the point of insertion on the antenna and they are more electron-dense and perpendicular to the surface of the antennae than the trichodea. There are four sensilla chaetica in each flagellomere, located in the equator, two on the laterals of the scaled area, and two on the scale free area (Fig 1).

3.2. Specificity and sensitivity of pheromone ORNs

3.2.1. ORN types

ORNs from sensilla trichodea can be categorized in three distinct groups based on their response to pheromone stimuli. One group of cells (called Z-cells) was very sensitive to the major pheromone component, *Z*8-12:Ac, and responded very little to the highest concentrations of *E*8-12:Ac and *Z*8-12:OH (Fig. 2). A second type of cells (called E-cells) was most sensitive to *E*8-12:Ac, showed an intermediate response to Z8-12:Ac, and responded very little to the highest concentrations of *Z*8-12:OH (Fig. 2). Z and E-cells made 63.6 % and 7.4% of the cells in sensilla trichodea, respectively (Table S3). The rest of the neurons (29%) did not respond to any of the three pheromone components, as determined with a single high-concentration puff, and they were considered as pheromone unresponsive ORNs (Table S3). Out of 176 sensilla sampled for their response to the three pheromone components we did not find a single ORN that responded to *Z*8-12:OH, with similar sensitivity as the Z- and E-cells to their own ligands.

Sensilla housing Z-cells were located along most of the length of the flagellomere (flagellomeres 10 to 35th), whereas sensilla housing E-cells were usually located in the distal mid-dorsal and proximal mid-ventral areas of the flagellomere. The proportion of the three cell types (Z, E and non-responding) was independent of whether they were on the scaled or on the scale-free area ($\chi^2 = 4.04$, df = 2, P > 0.132, Table S3).

3.2.2. Spontaneous activity and spike amplitude

More than half (62.5%) of the sensilla trichodea housed a single neuron of large spike amplitude (mean ± SEM, 1.81 ± 0.17 mV), whereas a smaller percentage

(21.02%) housed two neurons, where one was of large amplitude, similar to that of the single neurons, and the second one of a smaller spike amplitude, and 16.48% housed more than 2 neurons (Tables S3 and S4). When more than one neuron was present it often was the large and/or medium-amplitude neuron that responded to the pheromone stimuli (Table S4). The spike amplitude of Z- and E-cells was similar to each other and similar for single and paired Z- and E-cells (Table S4).

Z- and E-cells had similar spontaneous activity, and it did not differ between single and paired cells, but usually the smaller cells in paired or triplet groups had a higher spontaneous activity than the larger spike co-localised cells (Table S4). Unresponsive cells were not very different from Z- and E- cells in their spontaneous activity or spike amplitude parameters. Z-cells housed with other cells were significantly more abundant than singly housed Z-cells in the scale-free area than in the scaled area (Fisher exact test, $p < 0.001$) (Table S3).

3.2.3. Dose-response

The response of Z-cells to *Z*8-12:Ac and of E-cells to *E*8-12:Ac was sigmoidal in shape in the log-concentration scale, with a response intensity similar to hexane control up to the 1 pg stimulus concentration, rising steeply up to 1 ng stimulus concentration and starting to balance off at the 10 ng stimulus concentration (Fig. 2). Hexane produced minute changes in spontaneous activity. When the effect of the 0.38% contamination of *E*8-12:Ac in *Z*8-12:Ac was corrected, the response of E-cells to *Z*8-12:Ac decreased by about half, but it still was comparatively larger than the response of these cells to *Z*8-12:OH, or than the response of Z-cells to *E*8-12:Ac (Fig. 2).

Electroantennogram recordings showed significantly higher responses to the 3 pheromone components than to hexane at the highest concentration tested (1 μg;

planned contrasts between each compound and hexane following a GLM for each concentration, $P < 0.05$; Fig. S2). At the two lower concentrations tested, the two acetates stimulated the antennae more than hexane, but the response to the alcohol was similar to hexane.

3.2.4. Cross-adaptation

The dual response of E-cells to *E*8-12:Ac and *Z*8-12:Ac could result from a single neuron responding to the two compounds, or from two neurons (of identical spike amplitude) but each responding to a different isomer. A cross-adaptation test in E-cells, showed no response to the second puff in any of the cross-compound combinations tested, which is in agreement with the hypothesis that E-cells consist of just one cell responding to both isomers (Fig. 3). The cross-adaptation response was indistinguishable from the response to two consecutive puffs of the same isomer. In contrast, in Z-cells a puff of *Z*8-12:Ac following a puff of *E*8-12:Ac produced a response during the second puff, but not during the first puff, as is expected from a single cell responding just to *Z*8-12:Ac (Fig. 3).

3.2.5. Response duration of Z- and E-cells to *Z*8-12:Ac

Although both Z- and E-cells responded to *Z*8-12:Ac, the temporal pattern of response was different between them (Fig. 4). After stimulation the spike frequency increased from spontaneous activity levels to peak frequency, and then decreased to spontaneous activity levels in both cell types. The time after puff onset and half-rise was similar in Z-cells and E-cells (mean ± SEM, 0.148 ± 0.005 s and 0.147 ± 0.002 s, respectively; t-test, $p = 0.410$), but Z-cells remained active for a longer time as indicated by a longer time to half-fall (mean ± SEM, 0.299 ± 0.004 s) than in the E-cells (mean ± SEM, 0.224 ± 0.003 s; t-test, $p < 0.001$).

4. Discussion

4.1. Sensilla morphology

The six morphological sensilla types of *G. molesta* are similar to those reported in other tortricids (Wall, 1978; Ansebo et al., 2005; Razowski and Wojtusiak, 2004), and Lepidoptera in general (Hansson, 1998). The total number of sensilla trichodea that we recorded, however, is much lower than what George and Nagy (1984) report (4,382 and 9,095, respectively, for the sum of the two antennae). Nagy and George (1981) show that total counts of sensilla trichodea in *G. molesta* vary up to 30% among individuals reared under different conditions, so the different number of sensilla between studies could be related to this factor. The percentage of sensilla trichodea relative to other sensilla types that we observed is similar to the 81% reported by George and Nagy (1984).

Sensilla auricillica were the second most abundant sensilla type in the antennae of *G. molesta* males. Ebbinghouse et al. (1998) and Ansebo et al. (2005) distinguish two morphological types of sensilla auricillica in *C. pomonella*, however, although there was high inter-sensilla variability, we could not identify distinct sensilla auricillica classes in *G. molesta*. Sensilla auricillica in *C. pomonella* occur in medial or even proximal regions of the flagellomere (Ansebo et al., 2005), however we never found sensilla auricillica in the proximal half of the flagellomere of *G. molesta*.

Coeloconic sensilla made the third most abundant sensillar type in *G. molesta* males. The shape of these sensilla, with a central dome surrounded by a wall of michrotrichia, is rather characteristic. The size of sensilla coeloconica of *G. molesta* is similar to that of the small type sensilla coeloconica of *C. nigricana* (Wall, 1978). George and Nagy (1984) distinguish two types of sensilla basiconica in the antennae of *G. molesta* males,

however we could identify only one type. George and Nagy (1984) mapped the longer type I basiconica to the distal half of the scale-free area, and the shorter type II to the proximal half of the scaled area. Our sensilla basiconica were all located in the distal half of the flagellomere, and corresponded in length and number with sensilla basiconica type I of George and Nagy (1984), so they are probably the same type of sensillum. We, however, could not find sensilla basiconica type II of George and Nagy (1984), perhaps because it is shorter than sensilla basiconica type I.

Sensilla chaetica and styloconica served as landmarks due to their consistent number and position. The arrangement of four sensilla chaetica in the equator of the flagellomere and one sensillum styloconica at the tip is common in other tortricids (Wall, 1978; Ebbinghaus et al., 1997; Maher and Thiery, 2004; Ansebo et al., 2005).

4.2. Distribution of pheromone ORN types

Unlike what we expected the ORNs tuned to the major pheromone component *Z*8-12:Ac, are housed in different sensilla trichodea than the ORNs tuned to the stereoisomer, *E*8-12:Ac. Similar sensilla partition of ORNs is found in some noctuid moths, but in the other tortricids investigated, and many other moth species, major component ORNs share sensilla with minor component ORNs (reviewed in the following: De Bruyne and Baker, 2008; Baker et al., 2012). It has been proposed that the adaptive function of co-localized ORNs is related to the physiological constraint imposed by real time detection of precise odourant blend ratios (Baker et al., 1998; Baker et al., 2012; Binyameen et al., 2014). The question remains as to why in some species like *G. molesta*, pheromone ORNs are not co-localized, whereas in others like *O. nubilalis*, they share sensillum with other pheromone ORNs (Domingue et al., 2007).

As a general rule, when ORNs are co-localized the major component ORN has a larger dendrite size, whereas in single ORNs the major component ORNs, and their sensilla, are more abundant than the other neurons and their sensilla (Baker et al., 2012). The last rule applies to *G. molesta* because major and minor component ORNs occur in different sensilla, and the major component ORNs are more abundant than minor component ORNs, whereas the spike amplitude of both ORN types is relatively similar, which is an indication of similar dendrite size between them (Hansson et al., 1994).

4.3. Pheromone-unresponsive ORNs

A relatively large percentage (29%) of the sensilla trichodea in *G. molesta* males house ORNs that do not respond to any of the pheromone components. In male *Manduca sexta* (L.) 59 % of the sensilla trichodea host ORNs responsive to sex pheromone components, whereas the rest either respond to plant odours (19 %) or do not respond to any test compound (20 %) Kalinová et al. (2001). In male *A. segetum* (Hansson et al., 1989) and *H. subflexa* (Baker et al., 2004) relatively smaller percentages of unresponsive ORNs were reported in sensilla trichodea (11% and 2%, respectively). The short sensilla trichodea of *S. littorallis* females do not respond to any of 73 pheromone or plant ligands tested (Binyameen et al., 2012). Male *G. molesta* respond behaviourally to plant volatiles (Varela et al., 2011b; Il'ichev et al., 2009; Lu et al., 2012), so some of their unresponsive ORNs could be tuned to plant volatiles. In addition, ORNs unresponsive to pheromone compounds could be used to detect pheromone compounds from other species that inhibit male *G. molesta* response to the female pheromone, such as *Z*6-12:Ac and *Z*10-14:OH (Guerin et al., 1986; Tòth et al., 1991), as happens in other species (reviewed in De Bruyne and Baker, 2008).

4.3. Ligand specificity of pheromone ORNs

Male *G. molesta* behaviourally discriminate small variations in the ratio of the two acetate isomers (Baker and Cardé, 1979; Baker et al., 1981), so they should have a detection system that reports the relative abundance of the two isomers in the blend. In the majority of moths this is achieved by having specific receptors to each of the main pheromone components (reviewed by De Bruyne and Baker, 2008). However in *G. molesta* the ORNs tuned to the minor component (*E*8-12:Ac) also respond to the major component (*Z*8-12:Ac). The 0.38 % *E*8-12:Ac contamination in the *Z*8-12:Ac solution contributed only slightly to the unspecific response of the E-cells, as we demonstrated after subtracting its effect. The cross-adaptation test indicated that sensilla housing the E-cell did not house a second ORN responding to *Z*8-12:Ac, and confirms that the receptor for *E*8-12:Ac is a single, and not very specific, ORN.

Low specificity ORNs for key pheromone components have been scarcely reported in the literature (Takanashi et al., 2006; Domingue et al., 2008). In *O. furnacalis* (Guenée) a large proportion of the ORNs respond equally well to the two main components (*E*12-14:OAc and *Z*12-14:OAc) (Takanashi et al., 2006). However, unlike *G. molesta, O. furnacalis* also has ORNs specifically tuned to each pheromone component. So, how to explain the apparent absence of *E*8-12:Ac-specific ORNs in *G. molesta*? One possible explanation is that *E*8-12:Ac-specific ORNs are rare and were missed in our sample of 176 cells. Very low frequencies (< 2%) of ORNs tuned to pheromone components have been reported in other species (Hansson et al., 1990; Quero et al., 2004). Another possibility is that fully specific ORNs are not essential for pheromone blend discrimination in *G. molesta*.

To explain the last point we must assume that each ORN type (Z and E) innervates a different glomerulus, as happens in most moth species (reviewed by Lei and Vickers,

2008), both glomeruli will be excited by *Z*8-12:Ac, but more intensely the *Z*8-12:Ac than the *E*8-12:Ac glomerulus, due to the larger number of *Z*8-12:Ac ORN axons innervating it. Because *E*8-12:Ac will excite only the *E*8-12:Ac glomerulus, departures in the relative response of the two glomeruli with respect to excitation with *Z*8-12:Ac alone will inform the insect of the presence of *E*8-12:Ac in the blend. Differential glomerular excitation could, thus, report stimulus composition even when one of the two ORNs is not fully specific. To confirm this point we should determine the presence of specific glomeruli for the Z and E-ORNs. Male *G. molesta* has one large glomerulus at the entrance of the AL that is lacking in females (Valera et al., 2011a), so it is very likely that this glomerulus is innervated by *Z*8-12:Ac. Antennal retrograde staining coupled with electrophysiological recordings (Hansson et al., 1992) could confirm if each ORN type innervates a different glomerulus, and calcium imaging (Piñero et al., 2007b) could measure the relative ratio of response to different pheromone blends. Finally, different temporal response dynamics of Z and E-cells in response to *Z*8-12:Ac (longer lasting response in Z cells), could provide odour identity information to the brain, but how this could help the brain to discriminate between Z and E excitation in E cells is not clear.

Although it is generally accepted that pheromone receptors are highly specific (reviewed by De Bruyne and Baker, 2008), relatively few studies have determined the specificity of the most behaviourally relevant pheromone component receptors for a given species. Additional studies of receptor specificity are needed to determine if species bearing low-specificity pheromone receptors, like *O. furnicalis* and *G. molesta*, are more common than generally assumed.

4.4. Detection of Z8-12:OH

One unexpected result from our study was the absence of Z8-12:OH ORNs. In moths the ratio of pheromone compound receptors in the antenna roughly corresponds with the proportion of pheromone compounds in the pheromone blend (reviewed by Baker et al., 2012), and accordingly in *G. molesta* the ratio of *E*8-12:Ac to *Z*8-12:Ac in the pheromone blend (6:100, Baker et al., 1981) corresponds with the ratio of these receptors in the antennae (11.6:100, this study). *Z*8-12:OH makes about 10% of the pheromone blend of *G. molesta* (Linn and Roelofs, 1983), so we expected to find approximately one *Z*8-12:OH receptor for every 10 receptors of *Z*8-12:Ac, but we found none. Our EAG tests showed that the antenna perceives the 3 pheromone components, *E*8-12:Ac and *Z*8-12:OH with similar intensity, which demonstrates that *Z*8-12:OH stimulates some antennal ORNs, but it does not imply that these cells have a specific response to this compound. It is possible that the receptors for *Z*8-12:OH are found in very low numbers and that we may have missed them in our sampling of 176 ORNs, however the EAG test indicates that these receptors should be at least as numerous as those for *E*8-12:Ac. Likewise, sensilla other than trichodea may house receptors that would respond to *Z*8-12:OH, as the sensilla auricillica of *C. pomonella* which house ORNs that respond mainly to plant odours and to the sex pheromone (Ebbinghaus et al., 1998; Ansebo et al., 2005). Plant odours increase the response of *G. molesta* to the sex pheromone (Varela et al., 2011), so it is conceivable that *Z*8-12:OH stimulates ORNs that sense plant odours, and that this stimulation results in the behavioural synergism of the alcohol with the two acetates.

Whereas the role of the two acetates in male response is fairly consistent, that of the alcohol is less predictable. *Z*8-12:OH is found in female effluvia in a 22-30% ratio (Cardé et al., 1979; Baker et al., 1980), and it increases captures at 1% to 10% blend ratios (Cardé et al., 1975a; Baker and Cardé, 1979), supporting its role as a true

pheromone component. However, wide variations of *Z*8-12:OH in the blend do not have major effects on male response (Baker and Cardé, 1979; Linn and Roelofs, 1983, Linn et al., 1986), and in some locations *Z*8-12:OH is not produced by females (Lacey and Sanders, 1992), or does not seem to play a role in attraction (Jung et al., 2013), so it appears as if *Z*8-12:OH is not as crucial in the blend as the acetate mix, which may explain the absence of a *Z*8-12:OH receptor in our insects.

Interestingly, *Z*8-12:OH inhibits males of closely related species (*G. funebrana*, and *G. prunivora*) that use a similar ratio of the Z/E acetates as *G. molesta* (Baker and Cardé, 1979; Guerin et al., 1986), which suggests that a function of the alcohol in *G. molesta* could be to deter the attraction of other species. Furthermore, other alcohols affect the response of male *G. molesta* to the two acetates (Baker and Cardé, 1979; Cardé et al., 1975a; 1975b; 1979). The lack of *Z*8-12:OH specific receptors in our study warrants a reinvestigation of the role of this, and other alcohols, in the olfactory communication of *G. molesta*.

Acknowledgments

BA was supported by a Ph.D. fellowship from MINECO Ministry of Spain, and CG by research grant AGL2010-17486 from the same agency.

References

Ansebo, L., Ignell, R., Löfqvist, J., Hansson, B. S., 2005. Responses to sex pheromone and plant odours by olfactory receptor neurons housed in sensilla auricillica of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). J. Insect Physiol., 51, 1066-1074.

Baker, T.C., Cardé, R.T., 1979. Analysis of pheromone-mediated behaviour in male *Grapholita molesta*, the oriental fruit moth (Lepidoptera: Tortricidae). Environ. Entomol., 8, 956-968.

Baker, T.C., Cardé, R.T., Miller, J.R., 1980. Oriental fruit moth pheromone component emission rates measured after collection by glass-surface adsorption. J. Chem. Ecol., 6, 749-758.

Baker, T.C., Meyer, M., Roelofs, W.L., 1981. Sex pheromone dosage and blend specificity of response by Oriental fruit moth males. Entomol. Exp. Appl., 30, 269-279.

Baker, T.C., Hansson, B.S., Löfstedt, C., Löfqvist, J., 1988. Adaptation of antennal neurons in moths is associated with cessation of pheromone-mediated upwind flight. Proc. Nat. Acad. Sci. USA, 85, 9826-9830.

Baker, T.C., Ochieng, S.A., Cossé,. A.A., Lee, S.G., Todd, J.L., Quero, C., Vickers, N.J., 2004. A comparison of responses from olfactory receptor neurons of *Heliothis subflexa* and *Heliothis virescens* to components of their sex pheromone. J. Comp. Physiol. A, 190, 155-165.

Baker, T.C., Domingue, M.J, Myrick, A.J., 2012. Working range of stimulus flux transduction determines dendrite size and relative number of pheromone component receptor neurons in moths. Chem. Senses, 37, 299-313.

Beroza, M., Muschik, G. M., Gentry, C. R., 1973. Small proportion of opposite geometric isomer increases potency of synthetic pheromone of oriental fruit moth. Nature, 244, 149-150.

Binyameen, M., Anderson, P., Ignell, R., Seada, M.A., Hansson, B. S., Schlyter, F., 2012. Spatial organization of antennal olfactory sensory neurons in the female *Spodoptera littoralis* moth: Differences in sensitivity and temporal characteristics. Chem. Senses, 37, 613-629.

Binyameen, M., Jankuvová, J., Blaženec, M., Jakuš, R., Song, L., Schlyter, F., Andersson, M. N., 2014. Co-localisation of insect olfactory sensory cells improves the discrimination of closely separated odour sources. Funct. Ecol. DOI: 10.1111/1365-2435.12252

Byers, J. A. 2013. Modelling and regression analysis of semiochemical dose–response curves of insect antennal reception and behavior. J. Chem. Ecol., 39, 1081-1089.

Cardé, R.T., Baker, T.C., Roelofs, W.L., 1975a. Ethological function of components of a sex attractant system for Oriental fruit moth males, *Grapholita molesta* (Lepidoptera: Tortricidae). J. Chem. Ecol., 1,475-491.

Cardé, A.M., Baker, T.C., Cardé, R.T., 1979, Identification of a four component sex pheromone of the female oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae) J. Chem. Ecol., 5, 423-427.

Crawley, M. J. 2009. The R Book. John Wiley & Sons.

De Bruyne, M., Baker, T.C., 2008. Odor detection in insects: volatile codes. J. Chem. Ecol., 34, 882-897.

D'Errico, G., Faraone, N., Rotundo, G., Cristofaro, A. D., Trimble, R. M., 2013. Sensory adaptation of antennae and sex pheromone-mediated flight behavior in male oriental fruit moths (Leptidoptera: Tortricidae) after prolonged exposure to

single and tertiary blends of synthetic sex pheromone. Environ. Entomol., 42, 548-557.

Domingue, M.J., Musto, C.J., Linn Jr., C.E., Roelofs, W.L., Baker, T.C., 2007. Evidence of olfactory antagonistic imposition as a facilitator of evolutionary shifts in pheromone blend usage in *Ostrinia* spp. (Lepidoptera: Crambidae) J. Insect Physiol., 53, 488-496.

Domingue, M.J., Musto, C.J., Linn Jr., C.E., Roelofs, W.L., Baker, T.C., 2008. Olfactory neuron responsiveness and pheromone blend preference in hybrids between *Ostrinia furnacalis* and *Ostrinia nubilalis* (Lepidoptera: Crambidae) J. Insect Physiol., 54, 1261-1270.

Ebbinghaus, D., Loé sel, P.M., Lindemann, M., Scherkenbeck, J., Zebitz, P.W., 1998. Detection of major and minor sex pheromone components by male codling moth *Cydia pomonella* (Lepidoptera: Tortricidae). J. Insect Physiol., 44, 49-58.

Faraone, N., Errico, G. D., Caleca, V., Cristofaro, A. D., Trimble, R. M., 2013. Electrophysiological and behavioral responses of oriental fruit moth to the monoterpenoid citral alone and in combination with sex pheromone. Environ. Entomol., 42, 314-322.

George, J.A., Nagy, B.A.L., 1984, Morphology, distribution, and ultrastructural differences of sensilla trichodea and basiconica on the antennae of the oriental fruit moth, *Grapholitha molesta* (Busck) (Lepidoptera : Tortricidae). International J. Insect Morpho. Embryol., 13, 157-170.

Guerin, P. M., Arn, H., Buser, H. R., Charmillot, P., Tóth, M., Sziráki, G., 1986. Sex pheromone of *Grapholita funebrana* occurrence of Z-8- and Z-10-Tetradecenyl acetate as secondary components. J. Chem. Ecol. 12, 1361-1368.

Hansson, B.S., Van der Pers, J.N.C. Löfqvist, J., 1989. Comparison of male and female olfactory cell responses to pheromone compounds and plant volatiles in the turnip moth, *Agrotis segetum.* Physiol. Entomol., 14, 147-155.

Hansson, B.S., Tóth, M., Löfstedt, C., Szöcs, G., Subchev, M., Löfqvist, J., 1990. Pheromone variation among eastern European and a western Asian population of the turnip moth *Agrotis segetum*. J. Chem. Ecol., 16, 1611-1622.

Hansson, B.S., Ljungberg, H., Hallberg, E., Löfstedt, C., 1992. Functional specialization of olfactory glomeruli in a moth. Science, 256, 1313-1315.

Hansson, B.S., Hallberg, E., Löfstedt, C., Steinbrecht, R.A., 1994. Correlation between dendrite diameter and action potential amplitude in sex pheromone specific receptor neurons in male *Ostrinia nubilalis*. Tissue and Cell, 26, 503-512.

Hansson, B.S., 1998. Olfaction in Lepidoptera. Experientia, 51, 1003-1027.

Il'ichev, A.L., Kugimiya, S.,Williams, D.G., Takabayashi, J., 2009. Volatile compounds from young peach shoots attract males of oriental fruit moth in the field. J. Plant Interact., 4, 289-294.

Ivaldi-Sender, C., 1974. Techniques simples pour un e´levage permanent de la tordeuse orientale, *Grapholita molesta* (Lepidoptera : Tortricidae) sur milieu artificiel. Ann. Zool. Ecol. Anim., 6, 337-343.

Jung, C.R., Jung, J.K., Kim, Y. 2013. Effects of different sex pheromone compositions and host plants on the mating behavior of two *Grapholita* species. J. Asian-Pacific Entomol., 16: 507-512.

Kaissling, K.-E., Hildebrand, J.G., Tumlinson, J.H., 1989. Pheromone receptor cells in the male moth *Manduca sexta*. Arch. Insect Biochem. Physiol., 10, 273-279.

Kuhns, E.H., Pelz-Stelinski, K., Stelinski, L.L., 2012. Reduced mating success of female tortricid moths following intense pheromone auto-exposure varies with sophistication of mating system. J. Chem. Ecol., 38, 168-175.

Kalinová, B., Hoskovec, M., Liblikas, I., Unelius, C.R., Hansson, B.S., 2001. Detection of sex pheromone components in *Manduca sexta* (L.). Chem. Senses, 26, 1175-86.

Lacey, M.J., Sanders, C.J., 1992. Chemical composition of sex pheromone of oriental fruit moth and rates of release by individual female moths. J. Chem. Ecol., 18, 1421-1435.

Lei, H., Vickers, N., 2008. Central processing of natural odor mixtures in insects. J. Chem. Ecol., 34, 915-27.

Linn, C.E. Jr, Roelofs, W. L., 1981. Modification of sex pheromone blend discrimination in male Oriental fruit moths by pre-exposure to (*E*)-8-dodecenyl acetate. Physiol. Entomol., 6, 421-429.

Linn, C.E. Jr, Roelofs, W. L., 1983. Effect of varying proportions of the alcohol component on sex pheromone blend discrimination in male oriental fruit moths. Physiol. Entomol., 8, 291-306.

Linn, C.E. Jr, Campbell, M.G., Roelofs, W.L., 1986. Male moth sensitivity to multicomponent pheromones: critical role of female-released blend in determining the functional role of components and active space of the pheromone. J. Chem. Ecol., 12, 659-668.

Linn, C.E., Campbell, M.G., Roelofs, W.L., 1988. Temperature modulation of behavioural thresholds controlling male moth sex pheromone response specificity. Physiol. Entomol., 13, 59-67.

Lu, P.F., Huang, L.Q., Wang, C.Z., 2012. Identification and field evaluation of pear fruit volatiles attractive to the oriental fruit moth, *Cydia molesta*. J. Chem. Ecol, 38, 1003-1016.

Lu, P.F., Qiao, H.L., Xu, Z.C., Cheng, J., Zong, S.X., Luo, P.Q., 2013. Comparative analysis of peach and pear fruit volatiles attractive to the oriental fruit moth, *Cydia molesta*. J. Plant Interact., 1-8.

Maher, N., Thiery, D., 2004. Distribution of chemo- and mechanoreceptors on the tarsi and ovipositor of female European grapevine moth, *Lobesia botrana*. Entomol. Exp. Appl., 110, 135-143.

Molinari, F., Anfora, G., Schmidt, S., Villa, M., Ioriatti, C., Pasqualini, E., De Cristofaro, A., 2010. Olfactory activity of ethyl (*E, Z*)-2, 4-decadienoate on adult oriental fruit moths. Can. Entomol., 142, 481-488.

Nagy, B.A., George, J.A., 1981. Differences in the numbers of sensilla trichodea between reared and wild adults of the Oriental fruit moth, *Grapholitha molesta* (Lepidoptera: Tortricidae). Proc. Entomol. Soc. Ontario, 112, 62-72.

Najar-Rodriguez, A.J., Galizia, C.G., Stierle, J., Dorn, S., 2010. Behavioral and neurophysiological responses of an insect to changing ratios of constituents in host plant-derived volatile mixtures. J. Exp. Biol., 213, 3388-3397.

Najar-Rodriguez, A., Schneeberger, M., Bellutti, N., Dorn, S., 2012. Variation in attraction to host plant odors in an invasive moth has a genetic basis and is genetically negatively correlated with fecundity. Behav. Gen., 42, 687-697.

Najar-Rodriguez, A., Bellutti, N., Dorn, S., 2013. Larval performance of the oriental fruit moth across fruits from primary and secondary hosts. Physiol. Entomol., 38, 63-70.

Piñero, J.C., Dorn, S., 2007a. Synergism between aromatic compounds and green leaf volatiles derived from the host plant underlies female attraction in the oriental fruit moth. Entomol. Exp. Appl., 125, 185-194.

Piñero, J.C., Galizia, G.C., Dorn, S., 2007b. Synergistic behavioral responses of female oriental fruit moths (Lepidoptera : Tortricidae) to synthetic host plant-derived mixtures are mirrored by odor-evoked calcium activity in their antennal lobes. J. Insect Physiol., 54, 333-43.

Piñero, J. C., Dorn, S., 2009. Response of female oriental fruit moth to volatiles from apple and peach trees at three phenological stages. Entomol. Exp. Appl., 131, 67-74.

Quero, C., Bau, J., Guerrero, A., Renou, M., 2004. Responses of the olfactory receptor neurons of the corn stalk borer *Sesamia nonagrioides* to components of the pheromone blend and their inhibition by a trifluoromethyl ketone analogue of the main component. Pest Manag. Sci., 60, 719-26.

R Core Team. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/

Razowski, J., Wojtusiak, J., 2004. Some data on sensilla and sculpture of antenna in adult Tortricidae (Insecta : Lepidoptera). Genus, 15, 257-266.

Roelofs, W.L., Comeau, A., Selle, R., 1969. Sex pheromone of the Oriental fruit moth, Nature, 224, 723.

Rothschild, G.H.L., Vickers, R.A., 1991. Biology, ecology and control of the oriental fruit moth. In: Van der Geest LPS, Evenhus HH (eds) World crop pest, tortricid pests: their biology, natural enemies and control, vol 5. Elsevier, Amsterdam, pp 389-412

Stelinski, L.L., Gut, L.J., Miller, J.R., 2006. Orientational behaviors and EAG responses of male codling moth after exposure to synthetic sex pheromone from various dispensers. J. Chem. Ecol., 32, 1527-1538.

Takanashi, T., Ishikawa, Y., Anderson, P., Huang, Y., Löfstedt, C., Tatsuki, S., Hansson, B.S., 2006. Unusual response characteristics of pheromone-specific olfactory receptor neurons in the Asian corn borer moth *Ostrinia furnacalis*. J. Exp. Biol., 209, 4946-4956.

Tòth, M., Sziràki, G., Szöcs, G., Sàringer, E., 1991. A pheromone inhibitor for male *Grapholitha funebrana* Tr., and its use for increasing the specificity of the lure for *G. molesta* Busck (Lepidoptera : Tortricidae). Agric., Ecosyst. Environ., 35, 65-72.

Trimble, R.M., Marshall, D.B., 2010. Differences in the relationship between sensory adaptation of antennae and concentration of aerial pheromone in the oriental fruit moth and obliquebanded leafroller (Lepidoptera: Tortricidae): implications for the role of adaptation in sex pheromone-mediated mating disruption of these species. Environ. Entomol., 39, 625-632.

Trimble, R. M., 2012. Sexual behavior of *Grapholita molesta* and *Choristoneura rosaceana* (Lepidoptera: Tortricidae) in a flight tunnel after prolonged exposure to the aerial concentration of pheromone previously measured in orchards treated with pheromone for mating disruption. Environ. Entomol., 2012, 1481-1493.

Varela, N., Couton, L., Gemeno, C., Avilla, J., Rospars, J.P., Anton, S., 2009. Three-dimensional antennal lobe atlas of the oriental fruit moth, *Cydia molesta* (Busck) (Lepidoptera: Tortricidae): comparison of male and female glomerular organization. Cell Tissue Res., 337, 513-526.

Varela, N., Avilla, J., Gemeno, C., Anton, S., 2011a. Ordinary glomeruli in the antennal lobe of male and female tortricid moth *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) process sex pheromone and host-plant volatiles. The J Exp. Biol., 214, 637-645.

Varela, N., Avilla, J., Anton S., Gemeno, C., 2011b. Synergism of pheromone and host-plant volatile blends in the attraction of *Grapholita molesta* males. Entomol. Exp. Appl., 141, 114-122.

Wall, C., 1978. Morphology and histology of the antenna of *Cydia nigricana* (F.) (Lepidoptera: Tortricidae). International J. Insect Morph. Embryol., 7, 237-250.

Willis, M.A., Baker, T.C., 1988. Effects of varying sex pheromone component ratios on the zigzagging flight movements of the oriental fruit moth, *Grapholita molesta*. J. Insect Behav., 1, 357-371.

Willis, M.A., Baker, T.C., 1994. Behaviour of flying oriental fruit moth males during approach to sex pheromone sources. Physiol. Entomol., 19, 61-69.

Witzgall, P., Kirsch, P., Cork, A., 2010. Sex pheromones and their impact on pest management. J. Chem Ecol., 36, 80-100.

Figure captions

Figure 1. Distribution of sensilla types on the flagellum of *G. molesta* males. The top part of the graph shows SEM pictures of (A) the scale-free ventral region, (B) the dorsal region (which is normally covered with scales), and (C) the dorsal region when the scales have been removed. All sensillum types are seen on the scale-free ventral region (except the basiconica which are not visible in this SEM figure), whereas the scale-bearing dorsal region shows only sensilla trichodea and chaetica (B). All other sensillum types (except styloconica) are visible in the scaled area when the scales are removed (C). The bottom part of the graph shows a schematic representation of the number and position of the different sensilla types for a prototypical flagellomere. The dashed line in the scaled area indicates a protuberance on that flagellomere area. Sensilla auricillica (s.a), sensilla basiconica (s.b), sensilla chaetica (s.ch), sensilla coeloconica (s.co), sensilla styloconica (s.st.), and sensilla trichodea (s.t).

Figure 2. Response of pheromone ORNs of *G. molesta* males to 200 ms puffs of each of the three pheromone components at several concentrations. Dots show observed data (mean ± SEM) and lines the adjusted curves. **A)** In *Z*8-12:Ac ORNs, *E*8-12:Ac and *Z*8-12:OH produced minimal stimulation and were tested only at the low dosages. The response to *Z*8-12:Ac had sigmoidal shape in the log10 scale and was modelled with a non-linear regression (spike frequency = 8.1668+(57.42-8.17)/(1+exp((1.55-log10(pg))/0.73), r^2=0.99), where "pg" is the loading quantity of the stimulus in pg. N=33 for all the stimuli. Average response to hexane = 6.4 ± 1.2 spikes/s (mean ± SEM). **B)** *E*8-12:Ac ORNs responded strongly to *E*8-12:Ac, less intensely to *Z*8-12:Ac,

and almost no response to *Z*8-12:OH, which was tested only at the two highest concentrations. N=10-14 for all compounds. The response to *E*8-12:Ac was modelled (spike frequency = 50.57/(1+exp((1.69-log(pg))/0.59)), r^2= 0.99) and this equation was used to subtract the contribution of the 0.38% *E*8-12:Ac in *Z*8-12:Ac from the response of *E*8-12:Ac ORNs to *Z*8-12:Ac. The equations describing the response to Z8-12:Ac before and after correction are, respectively, spike frequency = 46.462/(1+exp((2.67-log10(pg))/0.61)), r^2= 0.99, and spike frequency = 21.47/(1+exp((1.98-log10(pg1))/0.46)), r2= 0.90, where "pg1" refers to the 0.38% *E*8-12:Ac in the amount of *Z*8-12:Ac shown in the x-axis. Average response to hexane = 0.14 ± 1.13 spikes/s (mean ± SEM). **C)** Representative recordings of one Z8-12:Ac ORN (top) and one E8-12:Ac ORN (bottom) stimulated with 1ng of each pheromone compound (200 ms puffs: horizontal bar over the trace).

Figure 3. Cross-adaptation test in *E*8-12:Ac ORNs (**A**, N=8) and Z8-12:Ac ORNs (**B**, N=10) in *G. molesta* males. ORNs were stimulated with two closely spaced puffs of *Z*8-12:Ac or *E*8-12:Ac (grey bars). The relative frequency of spikes in 25ms bins is plotted against time. Notice the lack of response to the second stimulation of the opposite stimulus in cross-stimulus tests with *E*8-12:Ac ORNs **(A)**, indicating that the same cell responds to both compounds. By contrast, in the *Z*8-12:Ac ORNs **(B)** stimulation with *Z*8-12:Ac adapts the cell to *Z*8-12:Ac but stimulation with *E*8-12:Ac does not, as is expected in highly specific ORNs. The loading quantities of *Z*8-12:Ac and *E*8-12:Ac in *E*8-12:Ac ORN were 100 pg and 1 ng, respectively, and in *E*8-12:Ac ORNs they were 100 pg and 1 ng, respectively. Only means are shown for the sake of clarity. **C)** Representative traces of the cross-stimulations (two top traces: one *E*8-12:Ac ORN; two

bottom traces: one *Z*8-12:Ac ORN). Each horizontal bar represents a 200 ms puff of either *Z*8-12:Ac or *E*8-12:Ac.

Figure 4. Response dynamics of *Z*8-12:Ac and *E*8-12:Ac ORNs to stimulation with *Z*8-12:Ac (100pg in *Z*8-12:Ac ORNs and 1ng in *E*8-12:Ac ORNs) and *E*8-12:Ac (100pg) (N=27-35). Stimulation with *Z*8-12:Ac resulted in a significantly longer-lasting response in *Z*8-12:Ac ORNs than in *E*8-12:Ac ORNs (t-test, $P < 0.05$). *E*8-12:Ac ORNs displayed similar dynamics to the two pheromone compounds.

Figure(s)

Figure 1

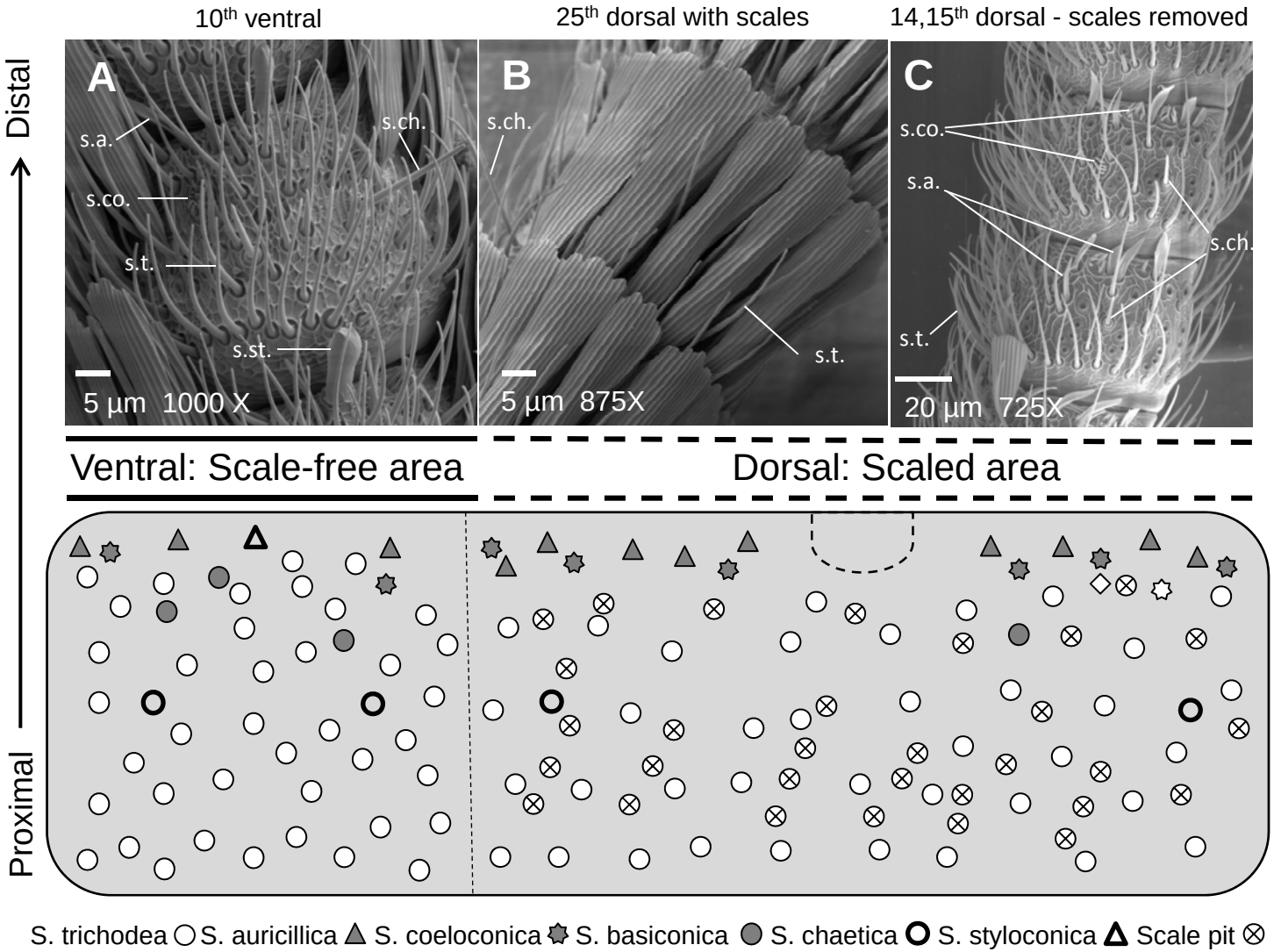


Figure 2

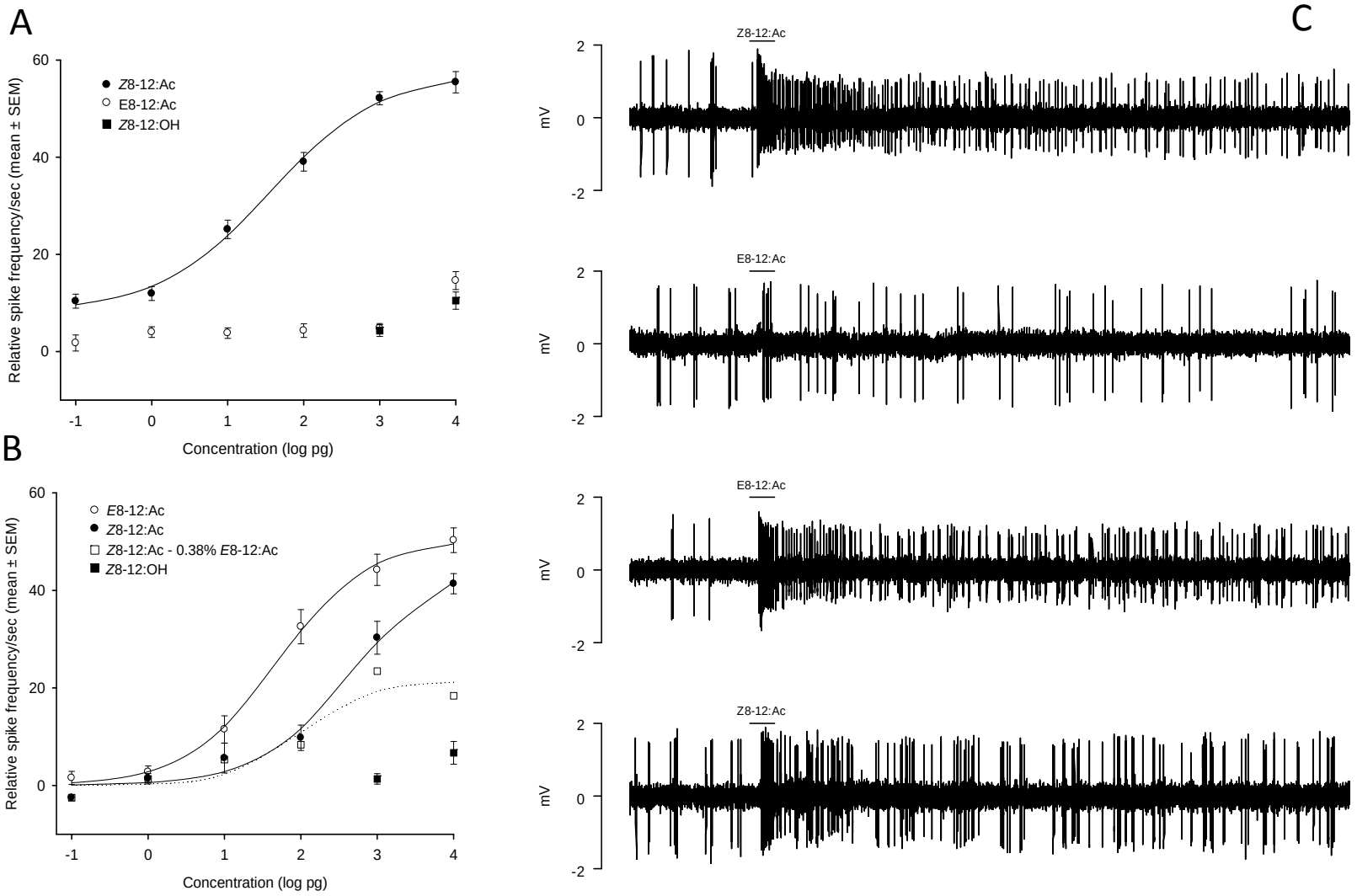

A
Relative spike frequency/sec (mean ± SEM)
Z8-12:Ac
E8-12:Ac
Z8-12:OH
Concentration (log pg)
B
E8-12:Ac
Z8-12:Ac
Z8-12:Ac - 0.38% E8-12:Ac
Z8-12:OH
Concentration (log pg)
C
Z8-12:Ac
E8-12:Ac
E8-12:Ac
Z8-12:Ac
mV

Figure 3

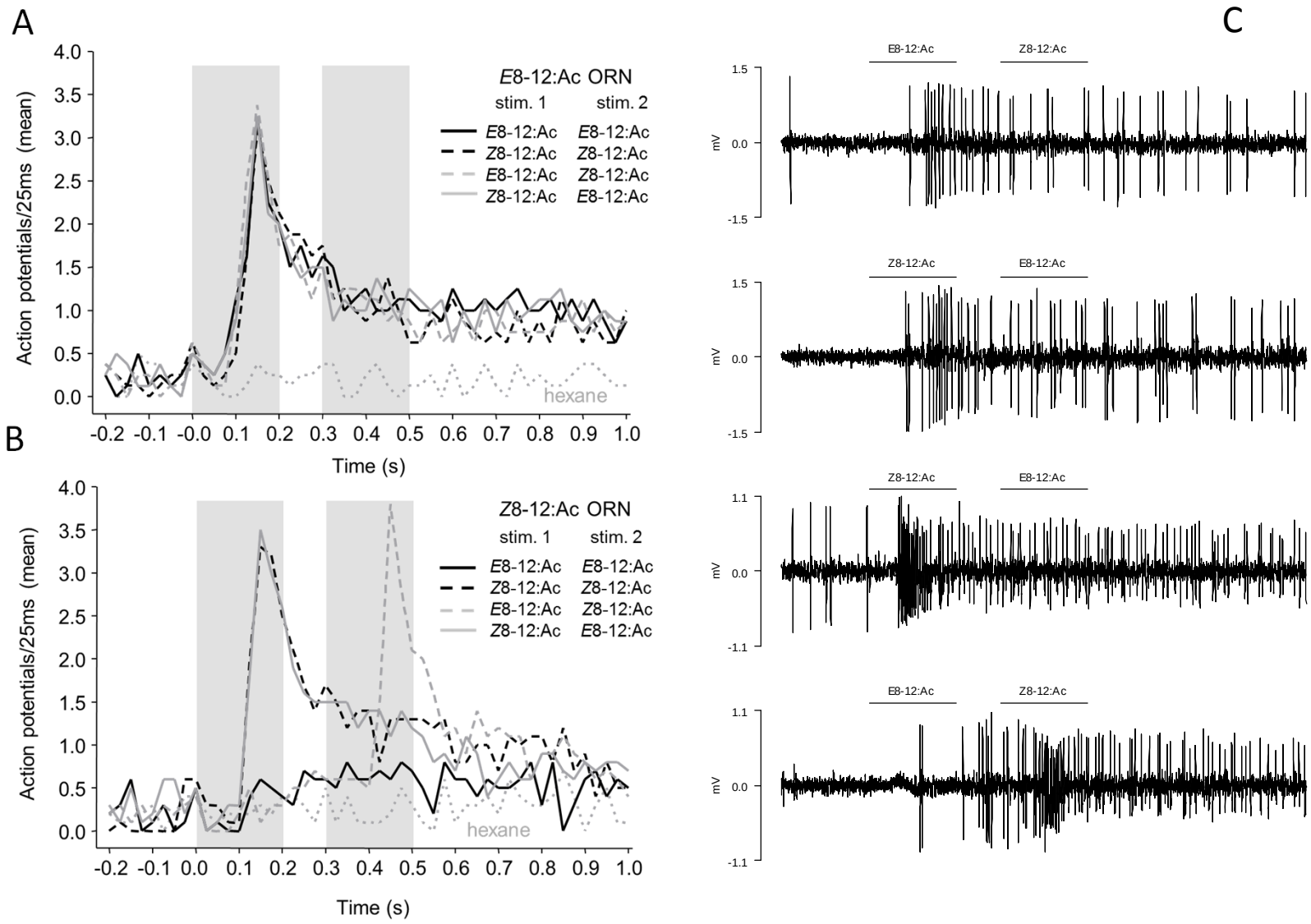
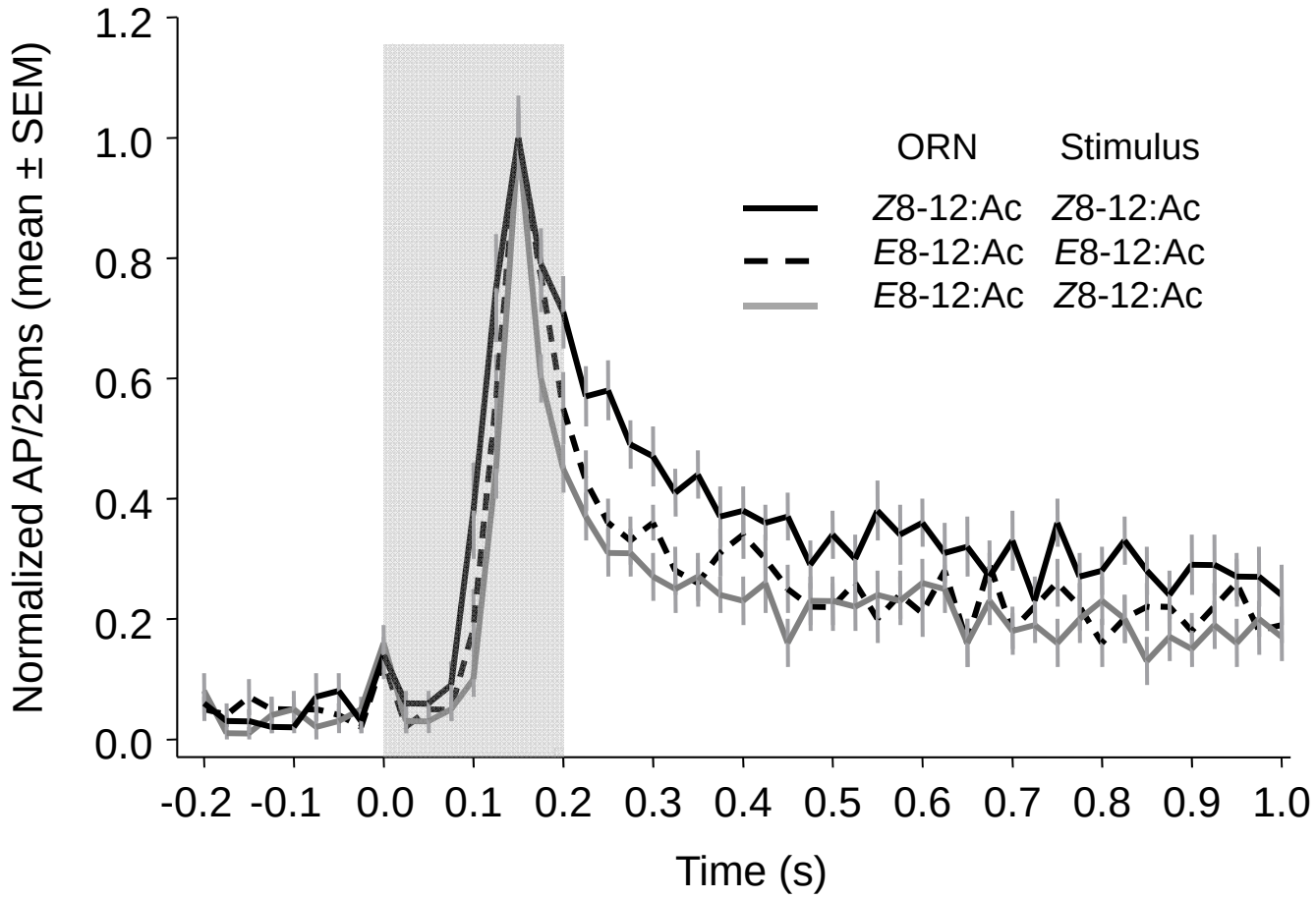

A
B
C
E8-12:Ac ORN
Z8-12:Ac ORN
stim. 1
stim. 2
E8-12:Ac
Z8-12:Ac
hexane
Action potentials/25ms (mean)
Time (s)
mV

Figure 4


Normalized AP/25ms (mean ± SEM)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Time (s)
ORN Stimulus
Z8-12:Ac Z8-12:Ac
E8-12:Ac E8-12:Ac
E8-12:Ac Z8-12:Ac

e-component

Click here to download e-component: Supplementary.docx